

RNA interference approach for clarifying the PPAR γ pathway using lentiviral vector expressing short hairpin RNA

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Abstract Peroxisome proliferator-activated receptor γ (PPAR γ) plays a central role in adipocyte differentiation and insulin sensitivity. Although PPAR γ also appears to regulate diverse cellular processes in other cell types such as lymphocytes, the detailed mechanisms remain unclear. In this study, we established a lentivirus-mediated short hairpin RNA expression system and identified a potent short hairpin RNA which suppresses PPAR γ expression, resulting in marked inhibition of preadipocyte-to-adipocyte differentiation in 3T3-L1 cells. Our PPAR γ -knock-down method will serve to clarify the PPAR γ pathway in various cell types in vivo and in vitro, and will facilitate the development of therapeutic applications for a variety of diseases. © 2004 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Peroxisome proliferator-activated receptor γ ; RNA interference; Short hairpin RNA; Lentiviral vector; Adipocyte

1. Introduction

The peroxisome proliferator-activated receptor (PPAR) family was discovered as an orphan nuclear receptor, and three different subtypes were subsequently identified, namely PPAR α , PPAR δ/β and PPAR γ . PPAR γ is abundantly expressed in adipose tissue and plays a key role in adipocyte differentiation and insulin sensitivity [1]. Recently, our group and other researchers reported that PPAR γ is also an attractive therapeutic target as it can play an important role in immune responses, especially in transcriptional regulation of inflammatory responses [2–5].

The biological role of PPAR γ had been widely investigated by using PPAR γ -deficient mice generated by targeted disruption of the PPAR γ gene. Since homozygous PPAR γ -deficient mice (PPAR $\gamma^{-/-}$) are embryonic lethal due to placental dysfunction [1], heterozygous mice (PPAR $\gamma^{+/-}$) have been used to investigate the role of PPAR γ in vivo experiments. However, PPAR $\gamma^{+/-}$ mice seem to be of limited use in some experiments, because PPAR γ also appears to regulate diverse cellular processes in cells that show lower levels of PPAR γ expression in comparison to adipose tissue [6,7].

RNA interference (RNAi) is a powerful technique for selectively silencing the expression of genes. Recent work has provided a system for the stable expression of short interfering RNA (siRNA) in mammalian cells, which is generally based on the expression of short hairpin RNA (shRNA) under the control of the RNA polymerase III promoter [8–11]. The technique has allowed for the development of a new approach for achieving targeted gene silencing of disease-associated genes in animal models as well as in cultured cells.

Lentiviral vectors (LVs) are a promising tool for exogenous gene transfer among gene transfer vehicles, because LVs have the advantages of infecting non-dividing cells and being stably integrated into the host genome resulting in long-term expression of transgene [12–16]. Furthermore, recent reports have demonstrated that virus-mediated RNAi could provide long-term silencing in mammalian cells [9,17,18]. In the present study, we attempted to develop a technique for suppressing the expression of PPAR γ in vivo and in vitro. We established a lentivirus-mediated shRNA expression system and identified a potent shRNA target sequence in the coding region of PPAR γ mRNA. This approach has enabled us to clarify a novel role of PPAR γ .

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2. Materials and methods

2.1. Vector construction

Vectors were constructed using standard cloning procedures. H1-RNA promoter was amplified from human genomic DNA (Clontech, Palo Alto, CA, USA) using the following primers: 5'-CCATGGAATTCGAACGCTGACGTC-3' and 5'-GCAAGCTTAGATCTGTGGTCTCATACAGAACTTATAAGATTCCC-3'. The amplified polymerase chain reaction (PCR) product was inserted into the *EcoRI*-*Bgl*II site of pHM5 [19], generating pHM5-H1. pHM5-H1 was designed to express shRNA upon the insertion of an appropriate sequence into the *Bgl*III/*Xba*I site (Fig. 1A). Oligonucleotides encoding

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Abbreviations: LV, lentiviral vector; shRNA, short hairpin RNA; MOI, multiplicity of infection; PPAR, peroxisome proliferator-activated receptor; GPDH, glycerol-3-phosphate dehydrogenase; BRL, rosiglitazone (BRL-49653)

3. Results and discussion

To develop an effective PPAR γ -knockdown method, we constructed an LV-based siRNA system in which shRNA encoding both strands of the targeting sequence is expressed under the control of human H1 promoter [24]. A human H1 promoter was cloned to generate pHM5-H1, and oligonucleotide encoding shRNA against PPAR γ mRNA was inserted (Fig. 1A). Subsequently, the cassette containing the H1 promoter plus the shRNA was transferred to the SIN LV construct (Fig. 1B). Using a shRNA target sequence against firefly luciferase, we previously demonstrated that our LV-based siRNA system effectively suppressed the target gene in mammalian cells (data not shown).

PPAR γ exists as two isoforms, termed PPAR γ 1 and PPAR γ 2,

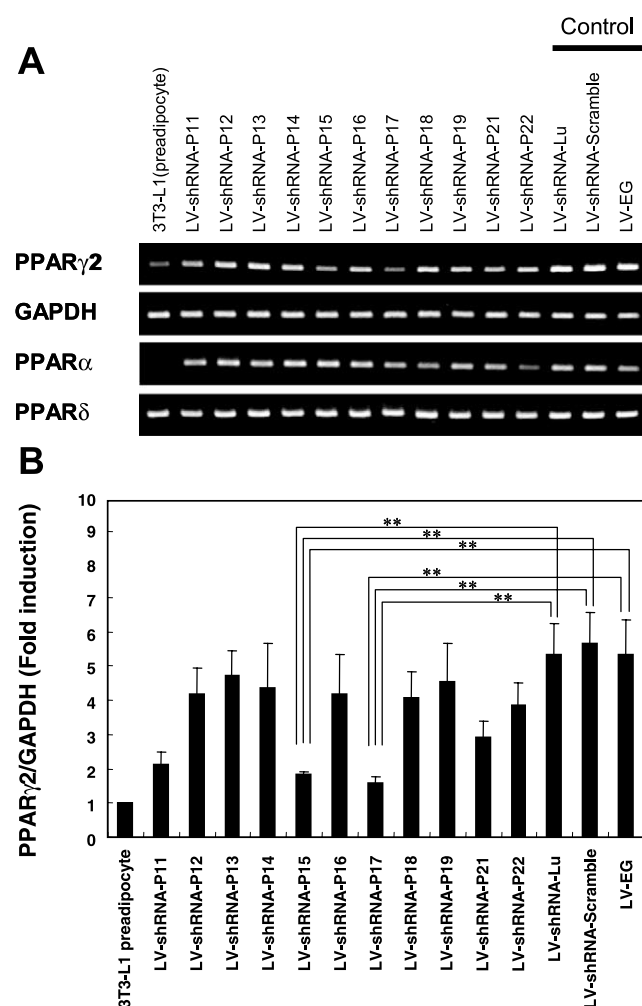


Fig. 2. Alteration of PPAR family mRNA levels in 3T3-L1 cells transduced with LV-shRNAs. A: 3T3-L1 preadipocytes were infected with each LV-shRNA (200 MOI) and then subjected to the differentiation protocol. Two days after the induction of adipocyte differentiation, mRNA levels of PPAR γ 2, PPAR α , PPAR δ , and GAPDH were determined by RT-PCR analysis. Results are representative gel images. B: Densitometric quantitation for PPAR γ and GAPDH from three to four independent experiments. Each PPAR γ value was normalized to the values for GAPDH and expressed as fold induction over the basal level detected in 3T3-L1 preadipocytes (bars, S.E.M.). ** $P < 0.01$ for LV-shRNA-P15 and -P17 compared with LV-shRNA-Lu, LV-shRNA-Scramble or LV-EG.

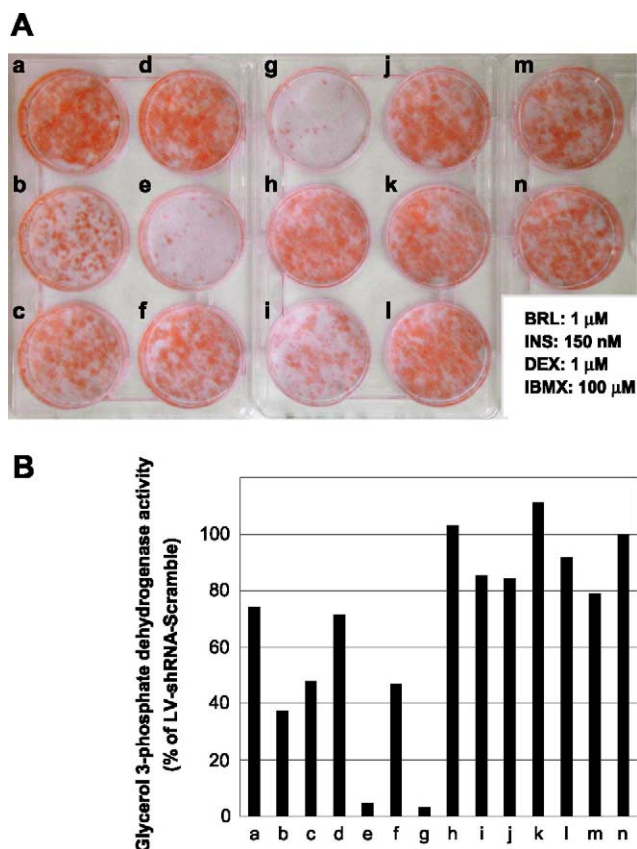


Fig. 3. Effect of LV-shRNAs on adipocyte differentiation. A: Differentiation of 3T3-L1 preadipocytes (infected with LV-shRNA; 200 MOI) to adipocytes was monitored by measurement of intracellular lipid accumulation using Oil red O staining on day 9. B: GPDH activity was measured on day 9. Data were expressed as percentage of the GPDH activity of 3T3-L1 cells which were infected with LV-shRNA-Scramble (200 MOI). a: LV-shRNA-P11; b: LV-shRNA-P12; c: LV-shRNA-P13; d: LV-shRNA-P14; e: LV-shRNA-P15; f: LV-shRNA-P16; g: LV-shRNA-P17; h: LV-shRNA-P18; i: LV-shRNA-P19; j: LV-shRNA-P21; k: LV-shRNA-P22; l: LV-EG; m: LV-shRNA-Lu; n: LV-shRNA-Scramble. Similar results were obtained in two independent experiments.

which are produced by a combination of different promoters and alternative splicing. PPAR γ 2 has an N-terminal extension of 30 amino acids and is very highly expressed in adipocytes [22,25]. We selected 11 target sequences in the coding region of PPAR γ mRNA and constructed LV-shRNAs against PPAR γ (Table 1). In the present study, LV-shRNA-Lu, LV-shRNA-Scramble and LV-EG were used as controls.

To find the most effective shRNA target sequence against PPAR γ , we analyzed the silencing of PPAR γ in 3T3-L1 cells during preadipocyte-to-adipocyte differentiation in which PPAR γ is known to be a master regulator of adipogenesis [1,26,27]. The expression of PPAR γ increases during the differentiation process and activation of PPAR γ protein by its ligand leads to adipogenesis through the activation of the adipogenic gene cascade. The 3T3-L1 preadipocytes transduced with each of the LV-shRNAs, i.e. 3T3-L1 cells expressing shRNAs, as listed in Table 1, were exposed to differentiation medium (DM) 2 days after confluence (day 0). Initially, silencing of PPAR γ expression was examined by RT-PCR after 2 days of culture in DM (Fig. 2). Although 3T3-L1 cells transduced with LV-shRNA-Lu, -Scramble and LV-EG showed

significant increases in the levels of PPAR γ mRNA, 3T3-L1 cells transduced with LV-shRNA-P15 and -P17 retained low levels of PPAR γ mRNA comparable to the level in preadipocytes maintained in normal culture medium. In contrast, the expression levels of GAPDH, PPAR α and PPAR δ were not altered by LV-shRNA-P15 or -P17. The other LV-shRNAs against PPAR γ caused moderate decreases in the levels of PPAR γ mRNA.

The differentiation of 3T3-L1 preadipocytes to adipocytes can be monitored by measurement of intracellular lipid accumulation and GPDH (an important enzyme in triglyceride

synthesis) activity [28–30]. Intracellular lipid accumulation was dramatically reduced in the LV-shRNA-P15- and -P17-infected 3T3-L1 cells as shown by Oil red O staining (Fig. 3A, e: LV-shRNA-P15; g: LV-shRNA-P17). GPDH activity also demonstrated that LV-shRNA-P15 and LV-shRNA-P17 express a potent shRNA which suppresses PPAR γ mRNA expression, resulting in marked inhibition of preadipocyte-to-adipocyte differentiation (Fig. 3B). We also confirmed that the expression of PPAR γ -inducible genes, such as uncoupling protein-1 and adipocyte fatty acid binding protein, were inhibited in 3T3-L1 cells transduced with LV-shRNA-P15 and LV-shRNA-P17 in the presence of the PPAR γ -specific ligand, BRL (unpublished data).

A recent study demonstrated that if the degree of complementarity to its target is reduced, siRNA can function as microRNAs which affect translational suppression without cleavage [31]. An important objective of this study was to determine whether the silencing effect of PPAR γ caused by these LV-shRNAs was specific for PPAR γ . In fact, several shRNA target sequences used in this study partially correspond to PPAR α or PPAR δ . Western blotting analysis demonstrated that PPAR γ protein levels were significantly decreased in the LV-shRNA-P15- and LV-shRNA-P17-infected 3T3-L1 cells, while LV-shRNAs did not alter the amount of PPAR α , PPAR δ or GAPDH protein (Fig. 4). These results were consistent with the result from RT-PCR analysis (Fig. 2).

Furthermore, we examined 3T3-L1 cells exposed to either LV-shRNA-Scramble, -P15 or -P17 by fluorescent microscopy for EGFP expression to identify cells not infected with those vectors, i.e. the 3T3-L1 cells not expressing the shRNA encoded by LV-shRNA-P15 or -P17 (Fig. 5). In the case of LV-shRNA-Scramble, which expresses control shRNA, the differentiation of preadipocytes to adipocytes was not affected by infection with LV. In contrast, all of the cells infected with LV-shRNA-P15 or -P17 retained their fibroblast-like morphology. Taken together, these results indicate that our LV-shRNA-based PPAR γ -knockdown method resulted in decreased PPAR γ expression and specific inhibition of the PPAR γ pathway, even in the case of adipocyte differentiation in which PPAR γ expression is strongly induced by DM and PPAR γ protein is effectively activated by the PPAR γ -specific ligand used in this study, BRL.

Accessibility of the siRNA might depend on the secondary structure of the target mRNA. However, a clear correlation between either secondary structure or GC content and effectiveness of target sites has not yet been recognized. Although we designed 11 different shRNAs against PPAR γ , we have not found any correlation between several factors that have been implicated in the accessibility of transcriptional/translational regulatory elements and effectiveness of target sites of shRNA until now.

In the present study, we developed a promising tool for suppressing the expression of PPAR γ . Our PPAR γ -knockdown method will serve to clarify the role of the PPAR γ pathway in various cell types in vivo and in vitro, and will facilitate the development of therapeutic applications for a variety of diseases.

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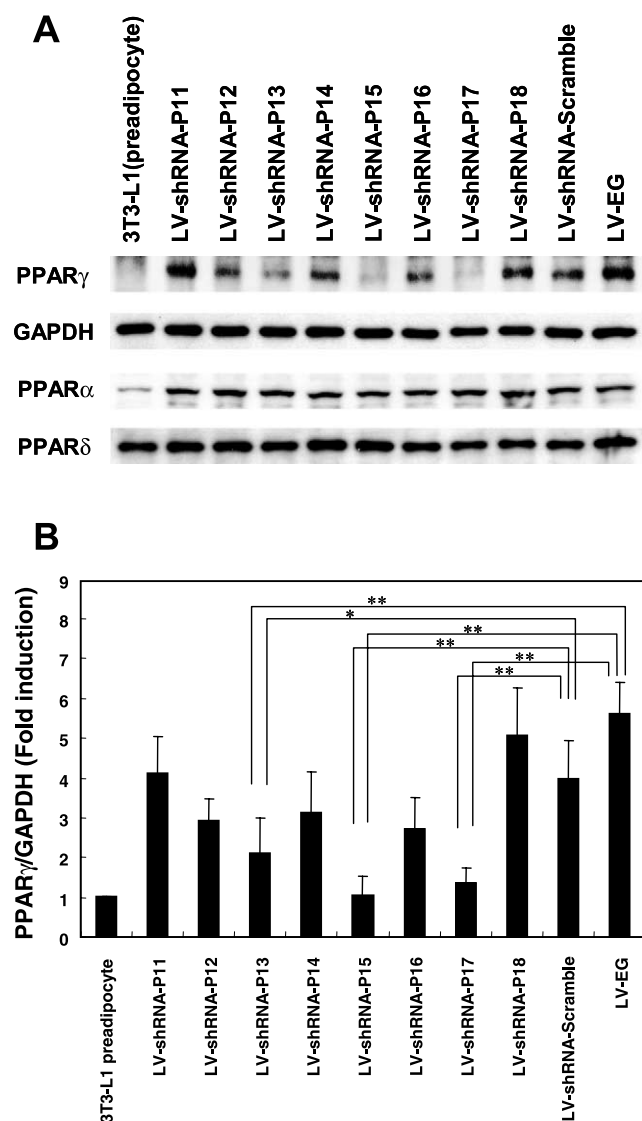


Fig. 4. Alteration of PPAR family protein levels in 3T3-L1 cells transduced with LV-shRNAs (200 MOI). A: Four days after the induction of adipocyte differentiation, the whole cell extract was analyzed by Western blotting with antibodies against PPAR γ , PPAR α , PPAR δ and GAPDH. Results are representative of three individual experiments. B: Densitometric quantitation for PPAR γ and GAPDH from three individual experiments. Each PPAR γ value was normalized to the values for GAPDH and expressed as fold induction over the basal level detected in 3T3-L1 preadipocytes (bars, S.E.M.). ** P < 0.01 for LV-shRNA-P13, -P15 and -P17 compared with LV-shRNA-Scramble or LV-EG. * P < 0.05 for LV-shRNA-P13 compared with LV-EG.

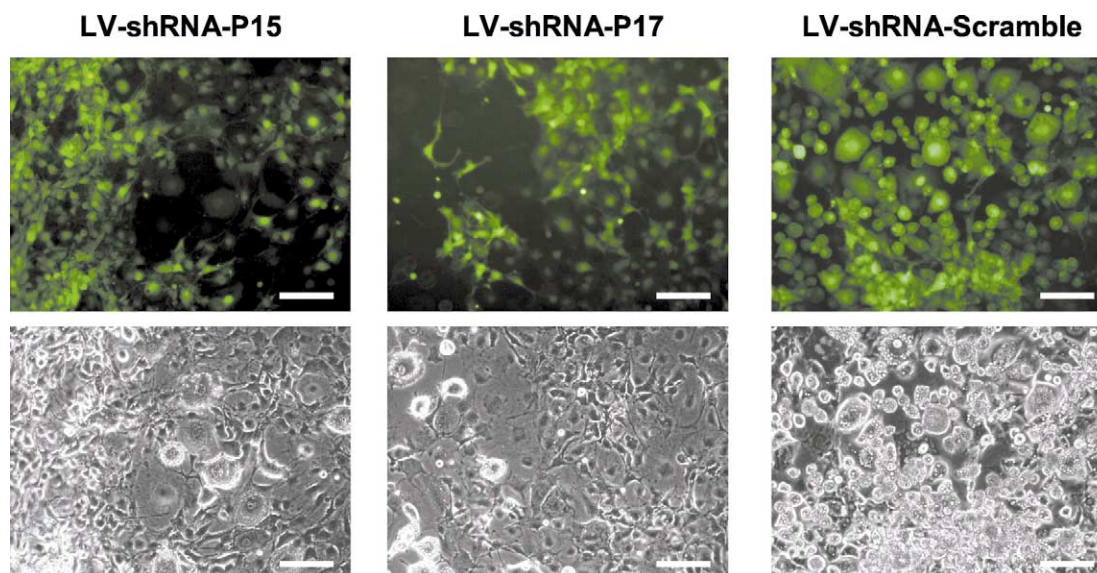


Fig. 5. Identification of the 3T3-L1 cells transduced with LV-shRNA. Brightfield and fluorescent microscopy images collected from the same field. The LV-shRNA-infected cells, which expressed EGFP, were detected as green fluorescence (upper panels) and morphologically identified mature adipocyte with a voluminous spherical shape and a large accumulation of intracytoplasmic lipid vesicles (lower panels). Bars represent 100 μ m.

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